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Synergistic interaction of soilborne plant pathogens and root-attacking insects in classical biological control of an exotic rangeland weed

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Abstract

This investigation of the possible role of pathogen–insect interactions on the mortality of *Euphorbia esula/virgata* plants was prompted by repeated observations of an apparent association in the field between damage to the roots of this plant caused by root-attacking insects and disease occurrence. In studies using microcosms in a greenhouse consisting of potted, caged plants of *E. esula/virgata*, combinations of *Fusarium oxysporum*, *Rhizoctonia solani*, or both fungi with adults and larvae of the flea beetle *Aphthona* spp. caused significantly greater rates of injury to *E. esula/virgata* than any single agent. Kaplan–Meier survival curves were used to examine the effects on time to mortality of combinations of various inoculum densities of *R. solani* per gram of air-dried soil with 0, 5, and 15 *Aphthona* per plant. At each insect level per plant, increasing inoculum density increased the rate of mortality of *E. esula/virgata*; the effect was significant at 5 and 15 beetles per plant using log rank tests. Additionally, at 5 flea beetles per plant, the rates of weed mortality in association with the second highest fungal inoculum concentration were similar to the mortality at the highest inoculum level, indicating that a minimum effective concentration is needed for effective synergism to exist. Cox regression analysis of proportional hazards was used to examine the relative contribution of plant pathogens and insects to weed mortality in the microcosms. The results indicated that plant pathogens are more than twice as likely to cause mortality of the target weed than insects under typical conditions and, under optimum conditions, are over four times more likely to do so. The results support the idea that supplementing flea beetle establishments with plant pathogens can be an effective means of both causing higher rates of successfully impacted release sites and greater biocontrol impact at individual release sites. Based on these findings it is recommended that a test of propensity for insect–plant pathogen synergisms should be a selection criterion for candidate agents. Additionally, it is recommended that survival analysis be applied to the target weed exposed to appropriate combinations of insects and pathogens as a means to assess the potential effectiveness of candidate agents. Application of one or both of these recommendations could increase success in classical biocontrol of weeds and reduce associated costs and environmental risks.
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1. Introduction

The earliest successful projects in biological control of weeds documented the effects of plant pathogens as a cause of mortality of the target weed (Dodd, 1940) or derived principles that specified the importance of plant pathogens (Wilson, 1943). Throughout the recent history of weed biocontrol, the importance of herbivore–plant pathogen interactions has been noted repeatedly (Caesar, 1996, 2000; Charudattan et al., 1978; Cullen, 1996; Wilson, 1969), with several authors emphasizing the promising yet unrealized potential of integrating insects and pathogens. The primary emphasis in agent selection throughout the history of weed biocontrol has been on insects. Despite this emphasis, approaches for selecting insects as biological control agents of weeds

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have remained unsettled (Blossey, 1995). Two current approaches are the selection of a set of insects to achieve simultaneous attack of different plant structures (Malecki et al., 1993) and the creation of cumulative stress through the introduction of multiple agents (Harris, 1981, 1984, 1991). Neither of these methods for selecting insects would necessarily lessen multiple introductions of ineffective agents, but could conceivably promote such an outcome. Although it is supposed that chances for success and degree of control are not adversely affected by the introduction of multiple species (Cock, 1986), a point of diminishing returns is approached and ecological risks are increased with each ineffective species introduced. The development of improved selection schemes aimed at reducing the number of agents to be introduced (Müller, 1990) is a valuable goal that has seldom been pursued. However, no current selection schemes for biocontrol agents has included an assessment of a propensity to incite or contribute to a synergistic interaction in order to rank or score the potential for success of candidate agents.

Recent studies on the impacts of insects as biological control agents of weeds have sought to to improve our understanding of the factors that affect the establishment of the agents (Freckleton, 2000; Shea and Possingham, 2000), but such studies have been rare. An equally important issue that has not been investigated is the failure to impact weed density subsequent to at least some degree of establishment of an insect. A review by the author of data on the establishment and impact of the flea beetles (*Aphthona* spp., Coleoptera: Chrysomelidae), released for control of the exotic rangeland invasive plant leafy spurge (*Euphorbia* spp., referred to herein as *Euphorbia esula/virgata*; see Gassmann and Schroeder, 1995; Lym and Carlson, 2002, for discussions of the taxonomy of this weed) on 439 stands, indicates that there are numerous sites with apparent insect establishment but with little or no measurable impact on the weed stand (Caesar, unpublished data). The data were accumulated over 12 years by the author's research group. This lack of impact appears to be a common problem. Such sites and those without detectable establishment of the insect comprise at least 66% of all *Aphthona* spp. release sites in the dataset. Recent summations of a smaller, more intensively managed and monitored set of 100 release sites of *Aphthona* spp. have indicated that after 5 years about 30% exhibit no impact of the introduced insect (D. Kazmer, University of Wyoming, personal communication). Given the considerable cost per introduced agent, research is needed on the means to ensure greater proportions of sites with successful impact. Research is also needed to identify likely synergists and to determine a "minimum effective dose" of the synergist or synergists. Additionally, accurate data are lacking on the levels of impact occurring at sites described as being successfully established. Thus, research is needed on factors that determine both establishment and impact.

The author and coworkers have shown that soilborne plant pathogens such as *Rhizoctonia solani* Kühn, *Fusarium* spp., and *Pythium* spp. are associated with insect damage caused by *Aphthona* and other species in US and Eurasian sites where *E. esula/virgata*, *Centaurea maculosa* Lam., and *Centaurea diffusa* Lam. occur (Caesar, 1995, 1996; Caesar et al., 1993, 1996a, 1998, 1999, 2002). Dead and dying plants within stands of these plants heavily impacted by root-attacking insects are typically infected with one or more of these plant pathogenic fungi. Individual isolates of the respective soilborne pathogens have been shown to be highly virulent and many possess narrow host ranges against cultivated species and native grasses (Caesar, 1994a,b, 2000). Against this background, the present study was undertaken with the goal of experimentally confirming the relative contribution of plant pathogens to weed mortality and determining whether there were levels of insects and plant pathogens at which a synergism was effective. Another objective was to examine whether there were indications of insect–pathogen synergisms in natural settings. This was studied by quantifying populations of *Fusarium* spp. in soil adjacent to roots of *Euphorbia* spp. in stands in the US, Europe, and Asia with and without detectable activity of root-feeding insects.

A fuller understanding of the mechanisms and interactions of the successful biological control achieved at several sites following insect releases may provide clues to ensuring success in a higher proportion of release sites. These findings could additionally serve to derive principles and approaches for selecting new agents, reduce costs and environmental risks, and help realize the potential of insect–pathogen interactions. Both the author (Caesar, 2000) and others (Hatcher and Paul, 2001) have underscored the need for careful investigation of this potential.

2. Materials and methods

2.1. Inoculum densities of Fusarium oxysporum and Rhizoctonia solani in the field

It was hypothesized that effective insect–pathogen synergisms on roots of *Euphorbia* spp. within stands of these species would be reflected in sustained high population densities of the synergistic fungal pathogens in soil. To examine this question, inoculum densities of *Fusarium* spp. and *R. solani* were determined at sites where *Euphorbia* spp. had been damaged by root-attacking *Aphthona* spp. (the species causing root damage at most sites), *Chamaesphecia* spp. (Lepidoptera: Sesiidae), or *Oberea erythrocephala* (Schrank) (Coleoptera: Cerambycidae). Rhizosphere soils and soils adjacent to

plants were sampled from four categories of leafy spurge or related *Euphorbia* spp. stands: (1) *E. esula/virgata* sites in the US without applied control measures; (2) sites in the US exhibiting diminishing *E. esula/virgata* stand densities following insect releases; (3) *Euphorbia* spp. (predominantly *E. esula/virgata*) sites in Eurasia without detectable insects; and (4) sites in Europe and Asia with adults or larvae of *Aphthona* spp. or the other host-specific, root-attacking insect species named above present on plants. Soil samples were taken carefully from within a volume 10-cm deep and 3 cm from roots and crowns of plants by using a trowel. Usually, soil samples taken adjacent to three plants were pooled for analysis. Roots and crowns of plants were examined for the presence of larvae or evidence of larval damage. For sites with insect presence, only rhizosphere soil from plants with apparent insect-caused root damage was processed further. The soil (100–150 g) was then weighed and placed in 1-liter flasks to which 300 ml of sterile distilled water and washes of soil adhering to the roots were then added. The flasks were shaken on a wrist-action shaker for 2 min and any floating organic matter was transferred to a 50-mesh (300 μm) sieve by pouring the suspension onto the sieve. This process was repeated three additional times with 1-min periods of shaking. The accumulated organic matter was washed from the sieve into 200 ml of 0.1% water agar with 50 ml of tap water. Fourfold serial dilutions were prepared and 0.1 ml aliquots were spotted onto plates of Ko and Hora (1971) and Nash and Snyder, 1962 (NS) media that had been dried for 1 h in a laminar flow hood with their covers removed. Inoculated plates were incubated at 24 °C for 48–72 h and examined for typical colonies of *Fusarium* and *Rhizoctonia* on both media. Suspected colonies of either species detected on the plates containing the highest dilutions were transferred to 1.5% water agar (WA) and acidified potato dextrose agar (APDA) and incubated at 24 °C.

2.2. Fungal inoculum production

Isolates were stored at −80 °C in 15% glycerol. Cultures of *R. solani* and *Fusarium* spp. were grown on potato dextrose agar (PDA) at 20–28 °C. For initial studies on the effect of combinations of plant pathogens and insects on plant development, an isolate of *Fusarium oxysporum* Schlectend.:Fr. (designated ND94-8) and an isolate of *R. solani* anastomosis group 4 (designated 95-6) were used that had been previously described as being pathogenic to leafy spurge (Caesar, 1994a, 1996). Mycelial disks (9-mm diameter) taken from the edge of 5-day-old cultures growing on APDA were used to inoculate liquid media. The *F. oxysporum* isolate ND94-8 was grown in a liquid medium containing 2% (w/v) Dietfiber (Lauhoff Grain, Danville, IL, USA), consisting of fiber, carbohydrates, protein, and trace minerals. The *R. solani* isolate ND95-6 was produced in a broth medium (Caesar, 1994a) consisting of 250 ml of peptone, sucrose, and yeast extract supplemented with frozen bean pods (van Bruggen and Arneson, 1985) for a total volume of 400 ml. The culture was then incubated at 20 ± 5 °C for 10–14 days as a stationary culture, with occasional shaking, at which time sclerotia and microsclerotia had formed.

The fungal inocula were thoroughly mixed into the appropriate potting mix and incubated for 6–10 days at 20–25 °C. The potting mixes were assayed for inoculum densities by plating fourfold dilutions of soil on WA amended with 100 μg/ml each of streptomycin and chloramphenicol (Abawi and Martin, 1985) for *R. solani* or NS medium for *F. oxysporum*. The most probable number (Halvorson and Ziegler, 1933) of colony-forming units (cfu) per gram of air-dried soil was determined using the computer program of Clark and Owens (1983).

For studies on inoculum concentration of *R. solani* and the number of insects, the fungus was grown at 20–28 °C in the liquid medium with 2% Dietfiber. Mycelial mat from the liquid culture was triturated in a blender for 30 s and added to potting media for pathogenicity assays. Infested soil was then serially diluted on a volume basis to achieve inoculum levels of 10 and 1% of the initial level. The most probable number of cfu per gram of air-dried soil was determined as described above.

2.3. Plant propagation

Rooted stem cuttings of leafy spurge propagated from plants collected at a single location in northeast Montana were used in all experiments except one. Plants that had attained a mass of ca. 30 g fresh weight or more were selected and planted, one plant per 21.5-cm diameter clay pot. Five pots per fungal isolate, which comprised a treatment, were kept in a greenhouse at 20–28 °C. Plants to be used in experiments were harvested, washed free of potting mix, and replanted into an appropriate soil mix. For studies on the effects of various combinations of *Rhizoctonia*, *Fusarium*, and the *Aphthona* flea beetles, a pasteurized potting medium consisting of 40% organic compost, 10% sand, 20% sphagnum peat moss, and 30% loam soil was used. In later studies on inoculum concentration of *R. solani* and the number of insects, inocula were added to a potting medium containing equal volumes of peat and vermiculite.

2.4. Combined effects of insects and plant pathogens on leafy spurge

Leafy spurge was planted, one per pot, into 10 pots (15-cm diameter by 15-cm tall, plastic) each of soil infested with *R. solani*, *F. oxysporum*, a combination of both fungi, or unamended soil mix. When soil was infested with both fungi, each species was applied at half the inoculum level used with a single fungus. Cages

consisting of nylon netting material, (32 mesh or 530-μm mesh openings) supported by an aluminum frame were placed over all pots and secured with a clamp to prevent escape of flea beetle adults. Adults of *Aphthona flava* Gillebeau, *Aphthona nigriscutis* Foudras, or *Aphthona czawalinae* Wiese were released, 15 per cage, into half of the caged pots containing *R. solani*, *F. oxysporum*, a combination of the two fungi, or uninfested soil. Thus, there were eight treatments: a single population level of (1) *R. solani*; (2) *F. oxysporum*; (3) both fungi each at half the population level of treatments 1 and 2; (4) uninfested soils; and treatments 5–8 which were the same series of treatments as in 1–4 but with the addition of insects. Treatments were arranged in a completely randomized design. The temperature in the greenhouse ranged from 28 to 30 °C.

Plant damage caused by insect injury or disease was assessed on a 0–6 rating scale: 0, no damage; 1, wilting; 2, as 1, but with chlorosis; 3, moderate wilting, chlorosis, and some tissue death; 4, persistent wilting, chlorosis, and death of one or more stems; 5, extensive wilting, chlorosis, and tissue death or top dieback with regrowth; and 6, death of plant (no regrowth). Individual plants were rated at 2- to 4-day intervals for 42 days. Treatments were compared using repeated-measures analysis (JMP, Versions 3 and 4, SAS Institute, Cary, NC). The principal analysis, using planned contrasts were used to test whether the presence of plant pathogens singly or in combination, in addition to insects, had an effect on leafy spurge injury ratings compared to either class of agent alone. Other planned contrasts ($P = 0.05$) examined whether a combination of *R. solani* and *F. oxysporum* caused more damage than either fungus alone when compared to the control. Prior to this, initial analyses were conducted for an overall test of whether or not the shapes of the response curves of each treatment were the same (parallelism effect), whether there were overall effects of treatments on damage ratings (levels effect), and when averaged overall treatments, whether the slope of the damage index was other than zero over the length of the experiments (flatness) (von Ende, 2001). Plants that died during the course of the study were examined for larval root damage, and larval presence near or in roots to assess whether mortality was associated with direct versus indirect interactions with plant pathogens. At the end of the test period, random samples of *E. esula/virgata* root tissue from each treatment were plated on WA or WA with streptomycin and chloramphenicol to detect the presence of the respective fungi used to infest soil.

2.5. Effects of different population densities of R. solani and Aphthona spp. on leafy spurge mortality

The Kaplan–Meier and Cox proportional hazards survival analysis algorithms (JMP 4, SAS Institute, Cary, NC) were used to access the effect of varying numbers of *Aphthona* and inoculum level of *R. solani* at the beginning of the experiment on survival of leafy spurge plants. Survival analysis is applied usually to individual survival times grouped according to treatment. The duration of exposure to the baseline factors until death of individual leafy spurge plants was recorded to the nearest 48 h, and confirmed by lack of regrowth until the experiment was terminated. For the year 2000 studies, this period was 26–120 days for individual plants and 30–91 days in 2001. Plants still alive at the end of the study were coded as censored.

In the present study, the nonparametric Kaplan–Meier procedure was used to obtain estimates of survival functions and survival curves of leafy spurge plants for all combinations of three inoculum levels of *R. solani*, an estimated 8, 0.8, and 0.08 cfu/g of air-dried soil mix, and unamended soil mix (designated 1.0, 0.1, 0.01, and 0, respectively) with 0, 5, and 15 adults of *Aphthona* spp. The Kaplan–Meier survival analysis procedure is commonly applied in public health, medical, and ecological research to analyze time to a specific outcome (often death) for individuals exposed to an explanatory factor (e.g., dietary fat or smoking in public health matter). Results plotted from this univariate survival analysis procedure were also inspected to determine whether there was a level of each agent below its respective maximum concentration (8 cfu of *R. solani* per gram of soil and 15 *Aphthona* adults per plant) which, in combination with the other agent, approached the combined effect of the maximum level of insect and pathogen. Such a level of each agent could be considered a ''minimum effective dose'' for each agent. Log rank and Wilcoxon tests were employed to test whether the survival functions were the same among treatments. The Cox proportional hazards regression method was used to assess the relative effects of plant pathogens or insects on weed mortality. This procedure was applied to obtain relative hazard ratios of mortality due to the baseline factors of soil inoculum levels of plant pathogens and numbers of adult insects per plant at the beginning of the experiment. The Cox method is a semiparametric procedure analogous to multiple regression. It does not assume any particular distribution of the data and is most often used to examine the effects of explanatory variables on survival times of subjects (*E. esula/virgata* plants in the present case). For both the Kaplan–Meier procedure and the Cox model, results from the initial two of the three greenhouse studies, beginning in summer 2000, were pooled for analysis. Data from the third study were analyzed separately and without the test for interaction between fungus and insects because the subject plants were generally of lower mass and overall vigor compared to plants used in the earlier studies. Plants that died were examined in the same manner as in the earlier experiment to assess

whether larval damage to roots had occurred and to detect *R. solani*.

3. Results

3.1. Soil populations of Fusarium spp. in association with roots and crowns of Euphorbia spp. in the US and Eurasia

Populations of *Fusarium* spp. in soil adjacent to roots and crowns of *E. esula/virgata* or *Euphorbia stepposa* Zoz exhibited a discernable pattern in relation to the presence or absence of insects based on data collected in the US, Europe, and Asia (Fig. 1). The log cfu of *Fusarium* spp. per gram of air-dried soil adjacent to plants within stands with *Aphthona*, *Chamaesphecia*, or *Oberea* spp. generally exceeded the numbers for plants from sites without insects. The upper range of populations of *Fusarium* spp. in soils from Eurasian stands of *Euphorbia* spp. with insect feeding exceeded that of other treatments. *Rhizoctonia* sp. was not detected from soils of any site category.

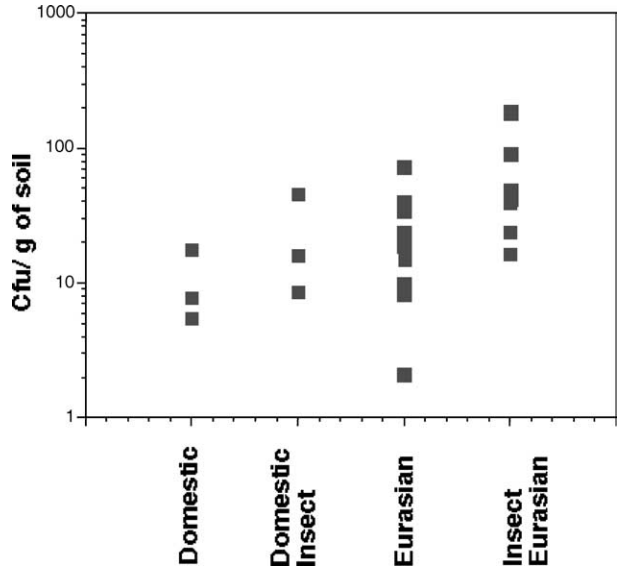

Fig. 1. Populations of *Fusarium* spp. in soil adjacent to roots of *Euphorbia* spp. from stands of the species in the US (Domestic) and Europe and Asia (Eurasia), with or without the activity of one or more of three root-feeding insect species. *X* axis, category of site sampled; *Y* axis, number of colony forming units of *Fusarium* spp.

3.2. Effects of combinations of R. solani, F. oxysporum, and Aphthona spp. on damage to leafy spurge in the greenhouse

Repeated-measures analysis of combined data from two studies of the same duration conducted in Sidney, MT in 1998 and 1999 indicated that plant pathogens combined with insects caused greater rates of damage to leafy spurge than any single agent. The repeated-measures analyses for tests of parallelism, flatness, and levels resulted in the rejection of applicable null hypotheses, indicating differences among treatments during the study (Fig. 2). Overall, the treatment-by-days interaction was significant at $P < 0001$. Planned contrasts (Table 1) showed that there was greater damage to leafy spurge in all cases between single or dual combinations of *R. solani* or *F. oxysporum* with or without *Aphthona* compared to *Aphthona* alone. The contrast between the *Aphthona*-alone treatment and the control was significant. Over the length of the study, neither plant pathogen alone caused significant damage to leafy spurge compared to the control, but the effect of *F. oxysporum* and *R. solani* combined was significant. The results of two other greenhouse studies, each of different duration conducted in Bozeman, MT in 1995 and Sidney, MT in 1997 were similar (data not shown). *Aphthona* larvae were consistently found in or near

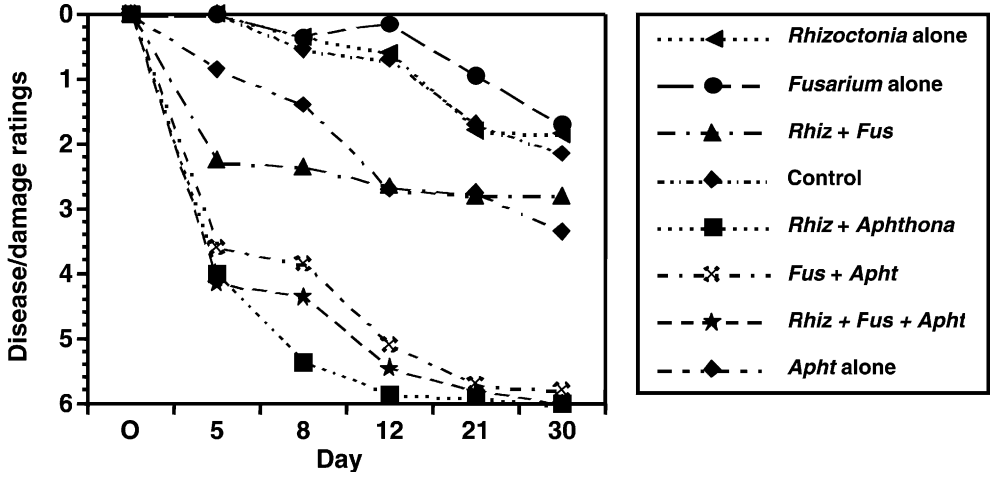

Fig. 2. Effects of *R. solani*, *F. oxysporum*, and both fungi used to infest soil with or without *Aphthona* spp. flea beetles on disease and damage to *E. esula* in greenhouse tests. R + F + A, *Rhizoctonia* (*Rhiz.*), *Fusarium* (*Fus.*), and *Aphthona*. Disease/damage ratings based on a 0–6 scale, 0, no disease/damage; 6, mortality.

Table 1
Comparisons of damage and disease caused by biocontrol agents on leafy spurge using planned contrasts[a]

Test[b]	*F* value	DF	$P > F$
Aphthona vs. control	10.5321	1	0.0018
F. oxysporum vs. control	1.1312	1	0.2911
R. solani vs. control	0.0744	1	0.7859
R. solani + *F. oxysporum* vs. control	10.7099	1	0.0016
Aphthona vs. *F. oxypsorum* + *Aphthona*	51.8357	1	<0.0001
Aphthona vs. *R. solani* + *Aphthona*	76.6357	1	<0.0001
Aphthona vs. *R. solani* + *F. oxysporum* + *Aphthona*	64.2859	1	<0.0001
Fungus vs. no fungus[c]	55.9	1	<0.0001

[a] The contrasts were used to examine the effects plant pathogenic fungi and root-attacking insects in the greenhouse. Plant damage caused by insect injury or disease was assessed by applying a 0–6 rating scale: 0, no damage; 1, wilting; 2, as 1, with chlorosis; 3, moderate wilting, chlorosis, some tissue death; 4, persistent wilting, chlorosis, death of one or more stems; 5, extensive wilting, chlorosis, tissue death or top die-back with regrowth; and 6, death of plant (no regrowth).

[b] Using repeated-measures analysis, the treatment-by-days interaction was significant ($P < 0.0001$).

[c] Includes all treatments with a single fungus or combinations of fungi with or without insects, compared to controls and *Aphthona* without fungi.

roots of dead or dying *E. esula/virgata* plants showing signs of insect feeding damage. Furthermore, the results of plating plant tissue showed consistent colonization by either fungus or combinations of both fungi. Thus, it was concluded that for appropriate treatments, mortality was due to *Aphthona* and plant pathogens used to infest the soil.

3.3. Effects of combinations of R. solani and Aphthona spp. on mortality of leafy spurge in the greenhouse

Kaplan–Meier estimates of survival curves clearly established that at each *Aphthona* population level per plant, increasing the inoculum density of *R. solani* increased the rate of leafy spurge mortality. The survival curves indicated the 1.0 and 0.1 *R. solani* inoculum concentrations caused the most rapid rates of mortality in combination with insects. This association of increasing *R. solani* inoculum density with differences in survival curves among treatments was significant ($P = 0.0043$, log rank test) at 5 flea beetles per plant (Fig. 3). The effect was greater and had a higher significance at 15 insects per plant ($P = 0.0004$, log rank test; Fig. 4). At 5 flea beetles per plant, rates of mortality for the 1.0 and 0.1 inoculum concentrations were similar (Fig. 3). Thus, the presence of even moderate

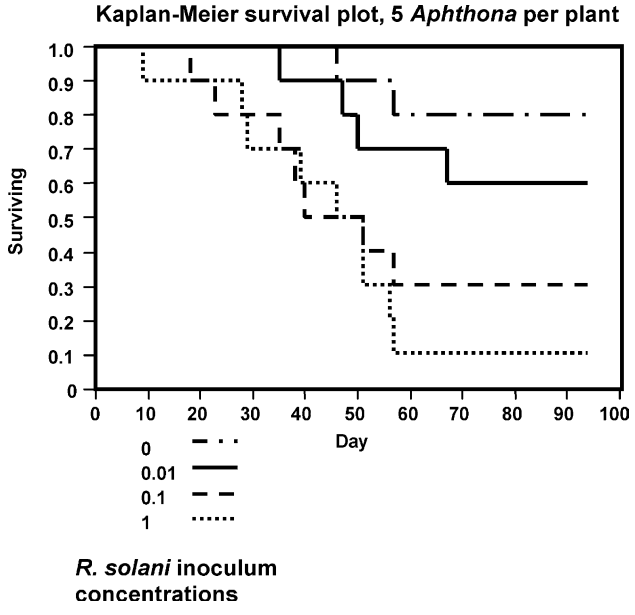

Fig. 3. Kaplan–Meier survival plot showing similarity in the rate of mortality of *E. esula/virgata* in soils with 1.0 and 0.1 soil inoculum concentrations of *R. solani* at 5 adults of *Aphthona* spp. per plant in microcosms in the greenhouse. *X* axis, days from beginning of experiment; *Y* axis, proportion of *E. esula/virgata* plants surviving.

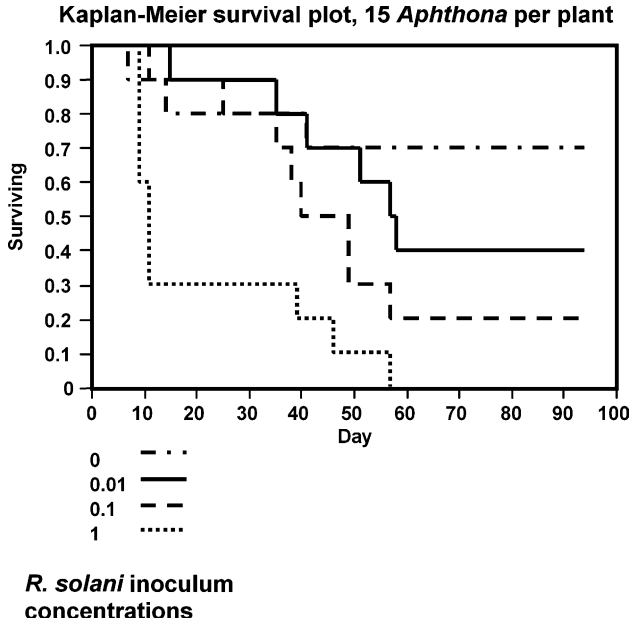

Fig. 4. Kaplan–Meier survival plot showing relative rates mortality of *E. esula/virgata* with 15 *Aphthona* spp. adults per plant and at various concentrations of *R. solani* in soil in microcosms in the greenhouse. *X* axis, days from beginning of experiment; *Y* axis, proportion of *E. esula/virgata* plants surviving.

Table 2
Hazard ratios for the effects of biocontrol agents on mortality estimated by the Cox regression model[a]

Source	Hazard ratio[a]	Lower confidence level	Upper confidence level	$P > \chi^2$
Rhizoctonia inoculum level[b]	2.505	1.641	3.758	0.0000
Insect level[c]	1.060	1.029	1.092	0.0002
Rhizoctonia inoculum level[b] × insect level	1.094	1.055	1.191	0.0369

[a] Data combined from both trials in 2000 and the effect assessed over all levels of respective agents. Hazard ratios > 1 indicate reduction in days to mortality of *E. esula/virgata*.
[b] Effect of increasing levels (8, 0.8, 0.08 cfu/g of air-dried soil mix) of *R. solani* inoculum density on mortality. Inoculum levels were adjusted by serial dilutions of soil.
[c] Effect of increasing number (5, 15) of *Aphthona* spp. adults on mortality.

Table 3
Hazard ratios for the effects of biocontrol agents on mortality estimated by the Cox regression model data assessing the effect over all levels of respective agents, third trial, 2001

Source	Hazard ratio[a]	Lower CL	Upper CL	$P > \chi^2$
Rhizoctonia inoculum level[b]	2.374	1.203	4.509	0.0137
Insect level[c]	1.043	0.996	1.090	0.0719

[a] Hazard ratios > 1 indicate reduction in days to mortality of *E. esula/virgata* due to the factor specified.
[b] Effect of increasing levels of *R. solani* inoculum density on mortality.
[c] Effect of increasing number of *Aphthona* spp. adults on mortality.

concentrations of the plant pathogen greatly increased the rate of mortality of leafy spurge in conjunction with a moderate number of insects per plant. Thus, an apparent synergism exists. The results of the Cox proportional hazards model (Table 2) show that *R. solani* inoculum density was more prognostic of mortality of leafy spurge, than the presence and number of insects. In the first two trials combined, the overall effect of *R. solani* in soil on mortality of leafy spurge was significant ($P = 0.0001$), as was the overall effect of insects ($P = 0.0002$). Furthermore, the interaction factor in the Cox model was significant ($P = 0.0369$), confirming the synergism between insects and pathogens apparent from the Kaplan–Meier plots. Hazard ratios greater than unity are indicative of reduced survival times of leafy spurge due to the respective agent, and the associated P value indicates the likelihood of the hazard ratio being different from unity due to chance. The overall hazard ratios due to the presence of plant pathogens were significant at 2.5 for the first two trials (Table 2) and 2.37 for the third trial (Table 3).

As in the experiments described in the previous section, the consistent presence of larvae in or near dead roots indicated that mortality was attributable to direct interactions of insect damage and disease.

4. Discussion

The main finding in this study is that the mortality of leafy spurge is due chiefly to plant pathogens contributing to a synergism with root-damaging insects. An earlier phase of the study had shown that insect–plant pathogen combinations caused accelerated damage/disease of leafy spurge plants compared to any single agent. These studies were undertaken to investigate an apparent correlation between successful stand reductions following the release of *Aphthona* spp. and the isolation of a set of soilborne pathogens from roots attacked by larvae of the flea beetle (Caesar et al., 1996a). The determination in the present study that there were intermediate levels of the insect and pathogen at which the synergism became operative (i.e., a minimum effective dose), is an important one. This indicates that synergism can be invoked without the presence of overwhelming populations of either agent. Thus, it is an attainable goal to supplement insect releases with plant pathogens for more rapid and consistent impact on weed density.

The results of the present study make a case for using the propensity for insect–pathogen synergism as a principal criterion in selecting biocontrol agents of weeds. According to a recent review, each weed biological agent tested and introduced requires three scientist-years which, with technical support and facilities, cost about $460,000 in 1997 (McFadyen, 1998). Had well-framed selection criteria been applied, the expenses of the introduction of as many as 18 agents that had been released for the biological control of leafy spurge might have been reduced, given that members of a single genus, *Aphthona*, accounted for nearly all of the impact on leafy spurge that has occurred. Addition of the potential for insect–pathogen synergism as a criterion to prerelease testing might have resulted in savings to current project and may do so in future projects. As

further support for the inclusion of this criterion in agent testing, recent results have shown that, as with leafy spurge, plant pathogenic soilborne fungi are associated with insect damaged roots and crowns of another perennial weed of rangelands, *C. maculosa*, in Europe (Caesar et al., 2002) and the US (Caesar, unpublished).

Over the relatively short duration of the present study, neither fungus alone caused significant damage when *E. esula/virgata* was exposed to them. Earlier studies had shown that the isolates used in the study caused significant levels of disease on *E. esula/virgata* (Caesar et al., 1998), including mortality in numerous instances. However, pathogenicity tests in those earlier studies were done over a longer duration than in the present study. This indicates that in testing for pathogenicity of soilborne pathogens to perennial weeds such as leafy spurge, tests should be done over a longer duration than with annual plants. Based on the author's experience from prior studies with leafy spurge (Caesar, 1996; Caesar et al., 1998), it is unlikely that transplant failure, an artifact, affected the outcome of the present study. This conclusion is supported by the failure of single pathogens to significantly damage the weed over the short term and at the levels of soil inoculum used in this study.

The integration of biocontrol agents into the larger context of integrated pest management (IPM) has been a major objective in numerous IPM programs. Increasing the effectiveness of the biocontrol component of integrated control may require a precise study of the mechanisms of action by biological control agents and factors affecting them. Within the context of leafy spurge biocontrol, failing to consider insect–pathogen interactions as a principal mechanism of impact can cause misdirection of resources. Greater emphasis on implementing insect–pathogen interactions to achieve consistent impacts should be a priority over further searches for new agents based on their prospective adaptability to host and climate. Previous work has shown that suitable agents for the biocontrol of leafy spurge were found outside the supposed optimal ecoclimatic and host–plant matching boundaries (Gassmann and Schroeder, 1995). Exploration for further *Aphthona* spp. is continuing despite significant impact on the stand density of leafy spurge in a variety of environments by individual species of this flea beetle. The premise for continued exploration is that additional species will fill environmental "niches" leading to a higher proportion of impacted stands of *E. esula/virgata*. An alternative approach is to supplement unimpacted sites with plant pathogen synergists. *R. solani* and *F. oxysporum* have been tested in the field as granular formulations and have shown to reduce stand density of leafy spurge (Caesar et al., 1996b). The development of protocols for selecting new agents based on understanding the mechanisms for impact that might be generally applicable, has been minimized in favor of expedience in release as a priority and in the belief that detailed prerelease studies constitute an unacceptable delay (Harris, 1991). However, the increasing scientific scrutiny on classical biological control (Louda, 2000) and increased regulatory focus accorded prospective new agents however dictate the need for more directed approaches to selecting effective agents.

The author's field surveys in Europe in 1992, 1995, 1998, and 2001 revealed that stands of leafy spurge generally were found at the same locations (for example, within an area of 20–30 m^2) each year with some exceptions (Caesar, unpublished). However, the precise location of small clusters of *Euphorbia* spp. plants within the overall stand were often found to vary 3–6 m between visits, indicating some turnover of plants. Also, there were no plants that were at least 3 years of age, judging by their visually estimated overall mass compared to *E. esula/virgata* plants of similar age observed in the field in North America. Most plants in European stands of *Euphorbia* spp. had no more than a single shoot per root mass, as described previously (Caesar et al., 1998). Thus, higher rhizosphere soil populations of *Fusarium* spp. are apparently due to a recurring cycle of insect attack on roots of *E. esula/virgata* and infection of damaged roots by the fungus, followed by death of the plants and release of inoculum into the soil. Although *Rhizoctonia* spp. infected insect-damaged *Euphorbia* roots, the fungus was not detectable in corresponding adjacent soil. Significantly, this could suggest a close relationship among *Euphorbia* spp., root-attacking insects specific to these hosts, and *Rhizoctonia* spp.

The upper range of the confidence interval for the hazard effect of the *R. solani* inoculum density (Tables 2, 3) indicates a greater potential effect on the fungus of variations in temperature, moisture, and other factors at microsites near and on root surfaces. This in turn may be indicative of how such variations may determine the severity of disease, and thus the influence of plant pathogens in combination with insects, on the mortality of *Euphorbia* spp. Thus, these findings would predict that under optimal conditions, the relative effect of *R. solani* vs. *Aphthona* spp. on mortality of *E. esula/virgata* can be up to four times greater than that of either type of agent alone. Detailed studies on the epidemiology of *Rhizoctonia* disease have shown that minor changes in soil physical conditions can determine the occurrence of parasitic invasion and epidemics (Bailey et al., 2000; Otten et al., 1999, 2001). While broad climate matching has been considered to be an accepted means of selecting promising agents (Cameron et al., 1993; Sutherst and Maywalk, 1999), the microenvironment, both physical and biotic, at or near the plant surface may in fact determine the level of impact by insects and plant pathogens on a target weed.

To the author's knowledge, survival analysis of the host plant has not been previously applied in selecting biological control agents for exotic, invasive weeds. Typically, in biological control of insects, the related procedure of life-table analysis is focused on the agent but even this line of analysis is seldom applied in selecting weed biocontrol agents (literature surveys by the author). Given the high cost per agent discussed above and the current lack of an empirical, mechanistic basis for selecting agents, the use of survival analysis of the target weed under varying inoculum densities of pathogens and insect population levels is recommended. For example, considering current programs of classical weed biocontrol, in which numerous herbivores are available (Schaffner et al., 2001; Tewksbury et al., 2002), the application of survival-analysis data could potentially result in considerable savings in time and cost and reduced ecological risks. In addition to death of the target weed as an outcome, other more subtle outcomes could alternatively be measured. For example, the phenological effects of an agent or agents on time to flowering or seed set could be another measure. A further advantage is that survival analysis would encourage consideration of other mortality factors along with insects. As discussed above, greater emphasis on prerelease studies may help define a clearer outcome to be sought following release of an agent.

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